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Use of *Salmonella typhimurium* TA 98, YG 1024 and YG 1021 and deconjugating enzymes for evaluating the mutagenicity from smokers' urine

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Abstract

Four smokers were chosen for their different smoking habits, and their declared cigarette consumption confirmed by urinary measurement of nicotine and its metabolites. The promutagenicity of their urine was evaluated by the Ames test, modified according to Kado et al. (Mutation Res., 31 (1983) 25–32) after extraction on XAD2 Amberlite resin. The different *Salmonella typhimurium* strains TA 98, YG 1021 and YG 1024 were compared to determine the presence of amino aromatic compounds in the urine of smokers of blond and black tobacco. The strain YG 1024 shows higher mutagenicity than TA 98 for extracts from the smoker's urine and more particularly from black tobacco smokers. In addition, the pretreatment of urine by external enzymatic systems (β-glucuronidase or arylsulfatase) reveals the presence in the urine of glucurono- and sulfoconjugated forms of promutagens, including amino aromatic compounds.

Keywords: Smoker; Urinary promutagen; Ames test; Urinary extraction; Beta-glucuronidase; Arylsulfatase

1. Introduction

Several aromatic amines, including the carcinogens *o*-toluidine, 2-naphthylamine and 4-aminobiphenyl have been found in tobacco smoke [1,2]. On the other hand nitroarenes, also known as carcinogens, have been detected to some extent in cigarette smoke condensates [3]. Aromatic amines, such as 4-aminobiphenyl and 2-naphthylamine used as industrial products, have been demonstrated to induce bladder cancer in occupationally exposed workers and in experimental animals [1,4].

It is known that nitro- or amino aromatic compounds require, respectively, the reduction or the oxidation of their functional groups to the corresponding hydroxylamines for exerting their mutagenicity. Then arylhydroxylamines are esterified by acetyl-CoA and *N*-hydroxylarylamine-*O*-acetyltransferase (OAT) present in bacterial cells [5]. Watanabe et al. [6] have developed two strains of *Salmonella typhimurium* with higher levels of nitroreductase or *O*-acetyltransferase. These two strains, YG 1021 and

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YG 1024, have been obtained by cloning the genes corresponding to the above-mentioned enzymes into pBR322 plasmids and introducing the cloned plasmid in *Salmonella typhimurium* TA 98 [15]. The strain YG 1024 has been found to be more sensitive in detecting promutagenicity in smokers' urine compared to the conventional strain TA 98 [7], while the strain YG 1021 did not show any increased sensitivity [8].

The goal of this study was to define if the nature of cigarettes (blond or black tobacco, full or light cigarette) may modify the mutagenic potency of cigarette smokers' urine towards the strains YG 1024 and YG 1021 of *Salmonella typhimurium* and so to define, through a biological approach, whether or not aromatic amines and nitroarenes are present in smokers' urine.

The action of β-glucuronidase and arylsulfatase on the mutagenic potency of smokers' urine was also evaluated, in order to study if nitro- or amino-aromatic derivatives could be conjugated in urine as glucurono- or sulfoconjugated forms.

2. Materials and methods

2.1. Chemicals and deconjugating enzymes

Acetone Rectapur, dimethylsulfoxide (DMSO) Suprapur and Amberlite XAD2 resin came from Prolabo. 4-Nitroquinoline oxide, 2-nitrofluorene, anthracene, benzo[*a*]pyrene, 3-methylcholanthrene, 2-aminofluorene and 2-aminoanthracene were provided by Sigma laboratories. Purified β-glucuronidase from *Escherichia coli* (200 IU/ml) and purified arylsulfatase from *Helix pomatia* (25 IU/ml) were purchased from Diagnostic Pasteur and Boehringer Mannheim Laboratories, respectively.

2.2. Subjects and characteristic of urine (Table 1)

The study was carried out with 5 healthy male volunteers, 1 non-smoker (NS) used as a control and 4 smokers having different smoking habits and coded BF, Bl, bF, and bl, for black full, black light, blond full, and blond light tobacco, respectively. The smokers had cigarette consumptions ranging from 12 to 40 cigarettes/day. All subjects answered a questionnaire concerning the type of cigarette smoked, their number, and specific relevant medications used.

Each urine sample was a pool of several mictions of the same individual collected at different periods of the working day. After collection, samples were stored deep frozen (−18°C) in polypropylene bottles until assay.

To confirm and estimate tobacco consumption and tobacco impregnation, nicotine and its pyridinic metabolites were evaluated by a simple colorimetric assay, based on the König reaction, described by Barlow et al. [9] using barbituric acid. This 'Barlow index' was expressed as 'cotinine-equivalent' and reported to the creatinine content of the urine (mmol of creatinine) to account for the influence of diuresis and reduce the variance of urinary cotinine measurements as described by Haddow et al. [10]. The urinary creatinine content was evaluated for each urine samples according to a kinetic method [11] with a multi-parametric auto-analyzer (IL 508, Instrumentation Laboratories, Lexington, MA).

Table 1
Parameters characterizing the smoking habits and the tobacco impregnation of four smokers and one non-smoker

Subject	Tobacco	Cigarette nicotine content (mg/cigarette)	Cigarette tar content (mg/cigarette)	Daily consumption (cigarettes/day)	'Barlow Index' (mg cotinine/mmol creatinine)
BF	black full	1.3	16.9	40	2.37
Bl	black light	0.86	9.8	15	0.99
bF	blond full	1.09	14.9	12	1.28
bl	blond light	0.7	9.6	20	1.95
NS	–	–	–	0	0.03

Table 2
Mutagenicity of 7 chemicals towards *Salmonella typhimurium* TA 98, YG 1021, and YG 1024 [a]

Chemicals	Amount (μg/plate)	Revertant colonies/plate [b]		
		TA 98	YG 1021	YG 1024
4-Nitroquinoline-oxide (−S9 mix)	0	37 ± 6	30 ± 1	61 ± 3
	0.025	110 ± 11	89 ± 6	79 ± 8
	0.05	269 ± 28	303 ± 7	265 ± 7
	0.1	458 ± 39	489 ± 5	507 ± 9
2-Nitrofluorene (−S9 mix)	0	37 ± 6	30 ± 1	61 ± 3
	0.00625	–	196 ± 13	372 ± 56
	0.0125	123 ± 7	490 ± 84	688 ± 28
	0.01875	–	–	420 ± 12
	0.025	274 ± 12	848 ± 28	–
	0.05	598 ± 16	–	–
	0.1	904 ± 32	–	–
Anthracene (+S9 mix)	0	39 ± 3	31 ± 5	59 ± 4
	1.25	45 ± 7	47 ± 9	77 ± 5
	2.5	48 ± 6	32 ± 5	63 ± 3
	3.75	57 ± 2	31 ± 2	68 ± 1
	5	39 ± 8	34 ± 3	75 ± 8
Benzo[*a*]pyrene (+S9 mix)	0	41 ± 4	35 ± 6	61 ± 4
	0.0625	–	83 ± 7	90 ± 7
	0.125	124 ± 5	114 ± 7	139 ± 5
	0.25	269 ± 13	287 ± 13	272 ± 6
	0.375	307 ± 10	275 ± 11	271 ± 10
3-Methyl-cholanthrene (+S9 mix)	0	41 ± 4	35 ± 6	61 ± 4
	0.0625	–	65 ± 3	81 ± 5
	0.094	87 ± 5	–	–
	0.125	110 ± 9	104 ± 9	118 ± 8
	0.25	129 ± 6	109 ± 13	157 ± 9
	0.375	98 ± 13	98 ± 5	188 ± 3
	0.5	71 ± 9	91 ± 10	196 ± 27
2-Aminofluorene (+S9 mix)	0	39 ± 3	31 ± 5	59 ± 4
	0.0125	–	–	218 ± 18
	0.025	53 ± 5	62 ± 6	460 ± 36
	0.0375	–	–	750 ± 34
	0.05	110 ± 9	108 ± 2	492 ± 20
	0.075	174 ± 5	135 ± 5	–
	0.125	288 ± 32	312 ± 9	–
	0.25	447 ± 49	442 ± 18	–
	0.375	584 ± 22	530 ± 14	–
2-Amino-anthracene (+S9 mix)	0	39 ± 3	31 ± 5	59 ± 4
	0.0125	–	–	394 ± 12
	0.025	175 ± 8	184 ± 6	912 ± 27
	0.0375	–	–	1550 ± 98
	0.05	283 ± 2	340 ± 4	–
	0.075	490 ± 4	491 ± 8	–
	0.125	1512 ± 28	1260 ± 36	–

[a] The Ames test was carried out according to Kado et al. [13] with or without S9 mix.
[b] All values are the average of 3 independent experiments with standard deviations; the number of spontaneous revertants has not been substracted from the total.

2.3. Urine extraction and mutagenicity assay

After thawing at room temperature, 100 ml of the urine samples was adjusted to pH 7 using a 0.1 N NaOH solution and filtered on 2.3 ml (1073 mg) of purified Amberlite XAD2 resin using the method developed by Yamasaki and Ames [12], at a filtration rate of 2.3 ml/min. The resin was washed with 10 ml of distilled water in order to withdraw the residual urine and histidine. The resin was eluted with 10 ml acetone Rectapur. The extract was then evaporated at 45°C under a nitrogen stream. The solid residues were dissolved in 400 μl DMSO before determination of mutagenicity. When not used on the same day, extracts were stored frozen (−18°C) until use.

The mutagenicity of the urinary extracts was determined using the Salmonella mutagenicity assay according to the method with liquid preincubation modified by Kado et al. [13] with strains TA 98, YG 1021 and YG 1024. *Salmonella typhimurium* strain TA 98 was kindly supplied by Dr. B.N. Ames (University of California, Berkeley, CA, USA) and the more recent strains YG 1021 and YG 1024 by Dr. P. Watanabe (National Institute of Hygienic Sciences, Tokyo, Japan). The S9 mix was prepared from aroclor 1254 induced rat liver homogenates following the procedure of Ames et al. [14].

Positive controls were realized for each experiment using benzo[*a*]pyrene with metabolic activation and 4-nitroquinoline (4NQO) without S9 mix. Negative controls were realized testing XAD2 extracts from 100 ml distilled water and XAD2 extracts from 100 ml of the non-smoker's urine.

2.4. Deconjugating assays and bacterial toxicity assessment

Three replicated assays were performed on the most mutagenic urine (BF). One hundred milliliters of urine, adjusted to pH 7 with NaOH 0.1 N, were incubated with β-glucuronidase (200 IU/100 ml) or arylsulfatase (1 IU/100 ml), respectively, for 0.5 h at 37°C.

After hydrolysis, the urine samples were filtered on XAD2 resin according to the procedure described before. The mutagenic activities of the hydrolyzed and non-hydrolyzed urine acetonic extracts were evaluated at the same time on *Salmonella typhimurium* strains TA 98 and YG 1024.

The bacterial toxicity of hydrolyzed extracts was assessed on strain TA 98. Different dilutions of bacterial solution were allowed to grow on Petri dishes in the absence or presence of urine extract hydrolyzed or not by β-glucuronidase at different doses. Bacterial colonies were counted on the most adequate dilution plate and the number obtained (N) was compared to the number of bacterial colonies on corresponding control plates without extract, treated or not, (N_0). The toxicity was expressed in percent of bacterial growth inhibition ($N/N_0 \times 100$). This toxicity assay was realized twice.

Table 3
Summary of mutagenicity of 7 chemicals towards *Salmonella typhimurium* strains TA 98, YG 1021, and YG 1024 [a]

Chemical	S9 mix	Revertants/μmol [b]		
		TA 98	YG 1021	YG 1024
4-Nitroquinoline-oxide	−	24.9 ± 1.6	27.7 ± 2.4 NS [c]	27.3 ± 2.6 NS
2-Nitrofluorene	−	42.6 ± 3.3	158.2 ± 11.1 [d]	227.9 ± 18.6 [d]
Anthracene	+	0	0	0
Benzo[*a*]pyrene	+	3.6 ± 0.3	4.0 ± 0.3 NS	3.3 ± 0.2 NS
3-Methylcholanthrene	+	2.0 ± 0.1	2.0 ± 0.2 NS	1.7 ± 0.2 NS
2-Aminofluorene	+	8.3 ± 0.5	9.6 ± 0.7 NS	101.4 ± 7.3 [d]
2-Aminoanthracene	+	30.2 ± 1.9	31.7 ± 0.4 NS	176.0 ± 15.2 [d]

[a] The Ames test was carried out according to Kado et al. [13] with or without S9 mix.
[b] All values were determined from the linear portion of the dose–response curves.
[c] NS, non-significant difference.
[d] $p < 0.001$ (result from a statistical test comparing mutagenicities by reference to TA98 strain).

2.5. Evaluation of promutagenicity and statistical analysis

The statistical analysis was performed using the BMDP statistical software. A chemical or extract was defined as mutagen when it produced at least a doubling of spontaneous revertants number and a reproductive dose–effect relationship. For each smoker, the mutagenicity was determined in the linear part of the dose–response curve and evaluated by the corresponding regression coefficient. The average results presented in this paper correspond to the mean (±SD) calculated with independent assays. The different slopes were statistically compared between the 3 strains.

3. Results

3.1. Reference mutagens

The activity of reference mutagens on the different *Salmonella typhimurium* strains (TA 98, YG 1021 and YG 1024) with or without S9 mix is presented in Tables 2 and 3. In this study, the mean number of spontaneous revertants is 39 ± 5 for the strain TA 98, 35 ± 9 for YG 1021 and 60 ± 4 for YG 1024.

Without metabolic activation, the mutagenicity of 4-NQO is not significantly different between the 3 strains. On the other hand, 2-nitrofluorene is, respectively, 4 and 5 times more mutagenic on YG 1021 and YG 1024 strains than on TA 98.

With S9 mix, the 3 strains present the same sensitivity towards polycyclic aromatic hydrocarbons. Anthracene is not mutagenic while benzo[*a*]pyrene and 3-methylcholanthrene show equivalent mutagenic potency for the 3 strains.

Aromatic amines (2-aminofluorene and 2-aminoanthracene) show no significant difference between mutagenicity results with TA 98 and YG 1021. However, the mutagenicity of these two amines is, respectively, 11 and 6 times higher for YG 1024 compared to TA 98 and YG 1021.

3.2. Smokers' urine extracts

3.2.1. Without enzymatic hydrolysis

None of the negative controls (blank and non-smoker urine extract) showed mutagenic activity with or without S9 mix. Without S9 mix, no mutagenic potency of smokers' urine extracts is observed whatever the 3 strains. Tables 4 and 5 respectively present the dose-dependent revertants number of the urinary extracts and resulting genotoxic activity of

Table 4
Mutagenicity of urine XAD2 extracts from four smokers and one non-smoker towards *Salmonella typhimurium* TA 98, YG 1021, and YG 1024 [a]

Subject	Extract (µl)	Revertant colonies/plate [b]		
		TA 98	YG 1021	YG 1024
BF	0	44 ± 9	51 ± 6	61 ± 5
	1.25	116 ± 14	91 ± 15	211 ± 9
	2.5	185 ± 27	182 ± 28	502 ± 18
	3.75	241 ± 13	250 ± 16	832 ± 27
	5	292 ± 5	295 ± 11	964 ± 16
	7.5	277 ± 37	304 ± 13	940 ± 46
	10	254 ± 32	320 ± 3	768 ± 15
Bf	0	50 ± 2	57 ± 4	65 ± 4
	1.25	77 ± 3	82 ± 3	158 ± 2
	2.5	109 ± 2	115 ± 6	259 ± 9
	3.75	140 ± 5	135 ± 13	342 ± 4
	5	168 ± 3	171 ± 4	392 ± 6
	7.5	209 ± 14	170 ± 2	358 ± 4
	10	217 ± 8	170 ± 9	308 ± 1
bF	0	45 ± 3	54 ± 3	63 ± 3
	1.25	54 ± 2	60 ± 1	76 ± 2
	2.5	65 ± 4	70 ± 2	94 ± 1
	3.75	74 ± 7	83 ± 6	121 ± 12
	5	82 ± 2	91 ± 11	132 ± 2
	7.5	88 ± 4	96 ± 2	153 ± 3
	10	79 ± 13	95 ± 4	146 ± 8
bf	0	47 ± 2	52 ± 3	64 ± 4
	1.25	59 ± 1	59 ± 1	82 ± 5
	2.5	70 ± 4	70 ± 4	103 ± 3
	3.75	79 ± 2	79 ± 2	120 ± 1
	5	88 ± 12	88 ± 1	141 ± 2
	7.5	98 ± 3	99 ± 2	142 ± 7
	10	104 ± 6	104 ± 9	138 ± 15
NS	0	52 ± 4	57 ± 2	59 ± 1
	1.25	51 ± 3	56 ± 5	63 ± 6
	2.5	54 ± 8	53 ± 7	60 ± 3
	3.75	46 ± 6	49 ± 1	57 ± 1
	5	43 ± 2	58 ± 2	55 ± 4
	7.5	52 ± 1	60 ± 1	58 ± 1
	10	55 ± 3	53 ± 2	64 ± 3

[a] The Ames test was carried out according to Kado et al. [13] with S9 mix.
[b] All values are the average of two independent experiments with standard deviation; the number of spontaneous revertants has not been substracted from the total.

Table 5
Summary of mutagenic activity of urine XAD2 extracts from 4 smokers and 1 non-smoker towards *Salmonella typhimurium* TA 98, YG 1021 and YG 1024 [a]

Subject	Revertants/ml of urine [b]		
	TA 98	YG 1021	YG 1024
bl	33 ± 1	29 ± 2 NS [c]	61 ± 5 [d]
BF	202 ± 5	205 ± 13 NS	764 ± 34 [d]
Bl	96 ± 6	89 ± 4 NS	299 ± 15 [d]
bF	29 ± 5	30 ± 2 NS	58 ± 3 [d]
NS	0	0	0

[a] The Ames test was carried out according to Kado et al. [13] with S9 mix.
[b] All values were determined from the linear portion of dose–response curves.
[c] NS, non-significant difference.
[d] $p < 0.001$ (result from a statistical test comparing mutagenicities by reference to TA98 strain).

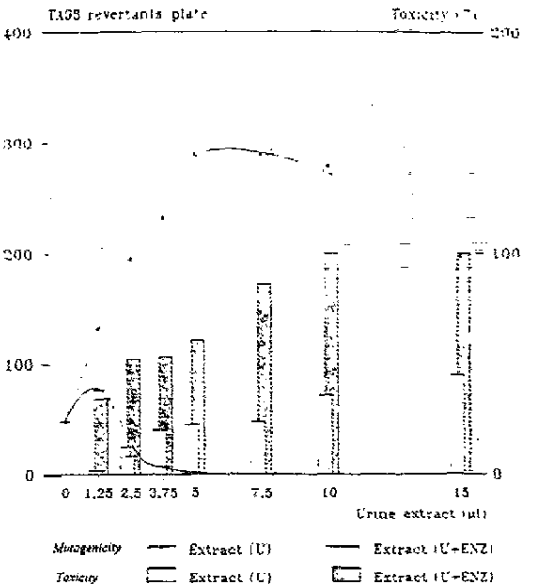

Fig. 1. Influence of β-glucuronidase on the mutagenicity and the toxicity of XAD2 extracts of the urine (U) of smoker BF. Hydrolysis by β-glucuronidase (200 IU/100 ml, 30 min, 37°C and pH 7). The Ames test was modified according to Kado et al. [13] with S9 mix and *Salmonella typhimurium* TA 98.

each smoker from an average of two replicated assays. Table 5 shows no difference in the sensitivity of the strains TA 98 and YG 1021 for the 4 different extracts, whereas the mutagenicity for YG 1024 is 2–4 times higher than that of the other strains. These differences of mutagenicity are greater for black tobacco than in blond tobacco smokers. The daily cigarette consumption alone cannot explain these differences in mutagenicity: in spite of a quasi identical consumption, the black light cigarette smoker's urine is 5 times more mutagenic than that of the blond light cigarette smoker.

3.2.2. With enzymatic hydrolysis (urine BF)

Controls realized with solutions containing only enzymes (either β-glucuronidase or arylsulfatase), submitted to the same extraction procedure as urine, are non-mutagenic and non-toxic for the strains. No mutagenic potency of treated urinary extracts is observed without S9 mix.

The hydrolysis of smoker's urine BF by β-glucuronidase leads to a decrease in the genotoxicity of the urinary extracts towards TA 98 (53%; $p < 0.001$) and a slight increase towards YG 1024 (20%; $p < 0.001$), as shown in Table 6. The toxicity test (Fig. 1) shows that the hydrolysis releases more cytotoxic compounds towards TA 98 decreasing the apparent genotoxicity.

On the other hand, arylsulfatase leads to an in-

Table 6
Influence of enzymatic treatment of the urine of BF smoker by β-glucuronidase or arylsulfatase on the mutagenicity of XAD2 extracts [a]

XAD2 extract	Revertants/ml urine	
	TA 98 + S9 mix	YG 1024 + S9 mix
Non-treated urine	204 ± 6	720 ± 6
Urine treated with β-glucuronidase (200 IU/100 ml)	108 ± 15 [b]	869 ± 10 [b]
Urine treated with arylsulfatase (1 IU/100 ml)	360 ± 16 [b]	1123 ± 14 [b]

[a] The Ames test was carried out according to Kado et al. [13] with S9 mix (mean of 3 assays).
[b] $p < 0.001$ (result from a statistical test comparing mutagenicities by reference to non-treated urine).

crease in the mutagenicity of urinary extracts for both TA 98 and YG 1024 strains (76% for TA 98 and 56% for YG 1024; $p < 0.001$) (Table 6).

4. Discussion and conclusion

Compared to TA 98, the strain YG 1024 is much more sensitive towards 2-aminofluorene and 2-aminoanthracene and YG 1021 and mainly YG 1024 are more sensitive to 2-nitrofluorene, as Einisto et al. [15] already demonstrated. We show no significant difference between the urinary extract mutagenicities with TA 98 and YG 1021, suggesting the absence of nitrofluorene and comparable bioactive compounds in smokers' urine, whatever their smoking habits. Previous studies showed the absence of nitro aromatic compounds as 1-nitronaphthalene, 1-nitropyrene, and 6-nitrochrysene, in cigarette smoke [1,2]. The absence of mutagenicity of urine extracts without metabolic activation confirmed the absence of these nitro derivatives. Indeed, numerous nitro derivatives (of benzo[*a*]pyrene, fluorene, naphthalene, etc.) are direct mutagens towards TA 98 [14,16,17].

The strain YG 1024 reveals higher genotoxicity than TA 98 for extracts from the 4 smokers' urine. The strain YG 1024 presents higher level of *o*-acetyltransferase (OAT) allowing an increase in its sensitivity to aminated or nitrated derivatives via esterification of the hydroxylaminated intermediates. Smokers' urine extracts therefore present some amino-aromatic compounds content and these compounds seem well concentrated by the XAD2–acetone procedure. Einisto et al. [8] also concluded to the presence of amino-aromatic compounds in smokers' urine concentrated on blue-cotton. Connor et al. [18] have already identified two TA 98 mutagens, 2-aminonaphthalene (β-naphthylamine) and one of its metabolites, 2-amino-7-naphthol, in the urine of a smoker whose mutagenicity was especially high. More recently, Peluso et al. [19] have given evidence of the presence of 2-amino-1-methyl-6-phenyl-imidazo(4,5-*b*)pyridine in black tobacco smokers' urine after analysis of ^{32}P adducts. They have already shown by this ^{32}P-postlabeling technique that amino-aromatic compounds were the major mutagens in smokers' urine [20].

In the present study, the genotoxicity increase of urinary XAD2 extracts towards the strain YG 1024 seems much higher for black cigarette smokers than for blond cigarette smokers, suggesting that blond cigarette smokers' urine contain less promutagenic aromatic amines than those of black cigarette smokers. In a previous paper [21], we have already shown that the urine of black tobacco smokers was more mutagenic than that of blond tobacco smokers, after adjusting for tobacco consumption. Bartsch et al. [22] showed that, for the same amount of smoking, black tobacco smokers excreted a 1.8-fold higher level of urinary mutagens than the blond tobacco consumers. Bryant et al. [23] and Bartsch et al. [22] found 1.5 times more 4-aminobiphenyl–hemoglobin adducts in the blood of black cigarette smokers than in the blood of blond cigarette smokers.

The deconjugation of smoker's urine shows the presence of glucurono- and sulfo-conjugated compounds becoming mutagenic after metabolic activation. Urinary extract obtained by β-glucuronidase hydrolysis is less mutagenic after hydrolysis towards TA 98 strains. It is explained by its increased cytotoxicity, confirmed by a toxicity test, which hides part of the mutagenicity. No indication is given on the nature of the compounds present in the smoker's urine: some authors [24] detected metabolites of a tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and its glucuronide in the smokers' urine. The treatment of urine by β-glucuronidase may increase the mutagenicity of the extracts towards YG 1024 by a liberation of aromatic aminated derivatives which are mutagenic and weakly cytotoxic.

On the other hand, the action of arylsulfatase on urine leads to an increase of the mutagenicity of urine extracts in both TA 98 and YG 1024 strains without increase of bacterial cytotoxicity showing that promutagens initially sulfo-conjugated are liberated after hydrolysis by arylsulfatase. The study with reference mutagens showed that both strains TA 98 and YG 1024 have similar behavior with all compounds, except aromatic amines and nitro aromatic compounds. Therefore, the highest increase of mutagenicity observed with YG 1024, in the presence of S9 mix, suggests that sulfo-conjugated aromatic amines are present in the smokers' urine.

Finally, the use of the two strains YG 1021 and

YG 1024 of *Salmonella typhimurium* allows the detection of amino aromatic promutagens in smokers' urine. The strain YG 1024 shows evidence of the presence of amino aromatic derivatives which might be responsible for bladder cancer [25]. Tobacco is a well-known risk factor for bladder cancer. These amino aromatic compounds are partly present in urine as glucurono- or sulfo-conjugated less genotoxic forms, but no further information could be obtained with these strains on the presence of nitrosamines or polycyclic aromatic compounds in smokers' urine, even if they are known to be present in tobacco smoke [1,26]. It would be interesting to associate physicochemical (for concentration and fractionation) and biological (for identification of bioactive fractions) approaches to have a better knowledge of the genotoxic compounds and their conjugates in the smoker's urine.

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